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Award Number: DAMD17-00-1-0223

TITLE: Effect of a Single Nucleotide Polymorphism (NP) on Breast Cancer Invasion

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REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20011127 067

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**

July 2001

3. REPORT TYPE AND DATES COVERED

Annual Summary (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE

Effect of a Single Nucleotide Polymorphism (NP) on Breast Cancer Invasion

5. FUNDING NUMBERS

DAMD17-00-1-0223

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REPORT NUMBER****9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) (*abstract should contain no proprietary or confidential information*) The lethality of breast cancer is derived from its ability to metastasize, and matrix metalloproteinases (MMPs) facilitate cellular invasion by degrading the extracellular matrix. One component of MMP regulation is transcription. We have identified a single nucleotide polymorphism (SNP) that enhances the transcriptional activity of MMP-1 promoters in transient transfections. The SNP consists of an extra guanine nucleotide that creates an ETS family transcription factor binding site (2G). We utilized cotransfection experiments, and determined that MMP-1 promoters containing the 2G polymorphism can interfere with transcriptional activity of promoters with the 1G polymorphism, but only at high concentrations of DNA. Analysis of five breast cancer cell lines with transient transfections reveals that the transcriptional effect of the 2G SNP is only observed in one cell line, and this cell line was the only one examined that expressed endogenous MMP-1. To better address the role of the polymorphism in endogenous gene expression, more samples are needed, and a source of those samples is human foreskin fibroblasts (HFFs). Initial experiments in HFFs have begun, but the amount of data currently limits statistical analysis. Future experiments include real time RT-PCR assays to better quantitate MMP-1 RNA in at least 50 HFF lines.

14. SUBJECT TERMS

Metastasis, SNP, Matrix metalloproteinase 1

15. NUMBER OF PAGES

13

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction:

The lethality of breast cancer is derived from its ability to metastasize, and a critical component of the metastatic mechanism is the degradation of extracellular matrix (ECM). Degradation of ECM is largely mediated by a family of proteins known as matrix metalloproteinases (MMPs). One subfamily of MMPs, the collagenases, are able to degrade stromal collagens type I, II, and III, and the most widely expressed collagenase is collagenase 1 (MMP-1). We have identified a single nucleotide polymorphism (SNP) that enhances the transcriptional activity of the MMP-1 promoter in tumor cell lines and normal stromal cells. The SNP consists of an additional guanine nucleotide creating an Ets transcription factor-binding site (5'-AGGA-3') at position -1607 in the MMP-1 promoter. When this 2G polymorphism is present, transcriptional activity from the MMP-1 promoter, as measured by transient transfections, is increased an average of 6 fold over the 1G polymorphism. A possible role for the SNP in cancer was suggested by observations of increased frequency of the 2G polymorphism in breast cancer cell lines and Japanese cancer patients as compared to normal DNA samples and non-patients(1, 2, 3). The MMP-1 gene is located on chromosome 11 (11q22.2-22.3) in a region associated with loss of heterozygosity in breast cancer patients. The studies outlined in this proposal are designed to characterize the mechanism of enhanced transcription from promoters with the 2G polymorphism, and to determine the effect of the polymorphism on transcription of the endogenous MMP-1 gene. We also hope to elucidate the role of the polymorphism and increased MMP-1 production in tumor invasion using a nude mouse model system. We propose that by understanding mechanisms leading to enhanced production of MMP-1 we can better understand the process of tumor invasion and metastasis, essential elements in the lethality of breast cancer.

Body:**Statement of Work**

Aim/Task 1: Using a variety of breast cancer cell lines (a) determine the levels of transcription of MMP-1 promoter DNA containing either the 1G or 2G allele, (b) characterize the DNA/protein interactions at these alleles, and (c) examine MMP-1 transcription in adriamycin sensitive vs. adriamycin-resistant breast cancer cells.

Months 1-6: Construct MMP-1 promoter/luciferase constructs containing 1G or 2G linked to either beetle luciferase or sea pansy luciferase, which are detected with different substrates.

Months 3-9: Begin testing constructs in breast cancer cell lines with transient transfections.

Months 9-18: Continue testing cell lines. Compare levels of transcription between adriamycin-sensitive and adriamycin-resistant cell lines. Compare with levels of

endogenous MMP-1 expression. Begin analysis of DNA/protein interactions with gel mobility shift assays.

Months 24-36: Finish transfections. Finish characterization of DNA/protein interactions. Analyze data for similarities/dissimilarities. Write PhD thesis.

Aim/Task 2: *Using a nude mouse model of human Metastatic breast cancer, and breast cancer lines that are homozygotic and heterozygotic for the 1G/2G/ alleles, (a) trace the development of metastatic tumors relative to their MMP-1 promoter genotype, loss of heterozygosity, location, size, and adriamycin sensitivity phenotype, and (b) examine excised tumors for MMP-1 expression by in-situ hybridization.*

Months 1-12: Begin studies to monitor the development of metastasis in nude mice injected with homozygotic and heterozygotic breast cancer cell lines. Inject 10^6 cells i.v. in tail (10 mice/group). Sacrifice after 4-6 weeks and look for Metastatic lesions in lungs and liver. Excise and size tumors, genotype them, examine for LOH, and begin *in situ* hybridizations for measuring MMP-1 expression.

Months 12-18: Continue with studies, and extend them to determine the earliest time at which a change in genotype may be detected. Sacrifice mice at earlier times and examine inguinal lymph nodes to determine if tumor cells are there and whether they have undergone a change in genotype. Continue *in situ* hybridizations.

Months 18-30: Compare the ability of different breast cancer cell lines (adriamycin-sensitive and adriamycin-resistant) to metastasize in nude mice and correlate with location, size, MMP-1 promoter genotype, and LOH. Finish *in situ* hybridizations.

Months 24-36: Analyze Data. Write PhD thesis.

Previously it had been discovered that DNA probes representing the MMP-1 promoter bound to nuclear proteins more tightly when they contained the 2G polymorphism(3). In the present study, initial experiments were designed to determine whether there was transcriptional competition at the MMP-1 promoter in cells that contained both the 1G and 2G polymorphism. In keeping with earlier experiments (3), I utilized the A2058 melanoma cell line which expresses high levels of MMP-1 constitutively. 4.3 kb of the MMP-1 promoter containing either the 1G or 2G SNP were cloned into vectors to drive the expression of either beetle or sea pansy luciferase. By using substrates specific to either the firefly or sea pansy luciferase I was able to cotransfect the 1G and 2G vectors into cells and measure the transcriptional activity from either promoter. Initially I measured the transcriptional activity from 0.5 μ g of 1G promoter in the presence of 0.5 μ g of either self (1G) or non-self (2G) competitor. That transcriptional activity was compared to the activity of the 1G promoter when no competitor was present. When no competitor DNA was added, experiments the DNA concentration was kept constant with pBluescript SK vector containing no promoter sequence. I repeated the experiments, measuring the transcriptional activity from 0.5 μ g of 2G promoter competed with self, non-self, and no competitor. The results of the

experiments indicate that at this concentration of DNA there is no difference in the ability of self or non-self to interfere with the transcriptional activity of either the 1G or 2G promoters (figure 1).

To examine if there would be transcriptional competition at higher concentrations of DNA, I determined the transcriptional activity from 2 µg of 1G promoter in the presence of 2 µg of either self competitor or non-self competitor. Once again the transcriptional activity was compared to the activity of 2µg of 1G promoter when no competitor was present. The experiment was repeated measuring the transcriptional activity of 2µg of 2G promoter in the presence of self, non-self, and no competitor. The results of the experiments indicate that there is a significantly greater ($p=0.026$) decrease in the transcriptional activity from 1G promoter DNA in the presence of 2G competitor when compared to the decrease in the presence of self (1G) competitor. However there was no difference in the ability of self or non-self to interfere with the transcriptional activity of 2 µg of 2G promoter (figure 2).

These cotransfection experiments were performed in A2058 melanoma cells to be consistent with the initial transfection experiments demonstrating a difference in the transcriptional activity of promoters containing the 1G and 2G SNPs(3). The conclusion that can be drawn from these experiments is that the promoters with the 2G polymorphism can out compete promoters containing the 1G polymorphism, but only at high concentrations of DNA. It is likely that at lower concentrations of DNA, the transcription factors are in excess, and therefore, a higher binding affinity of the 2G promoter for nuclear proteins does not influence transcriptional activity of the 1G promoter. These results add a functional component to earlier findings that DNA probes bind nuclear proteins stronger when they contain the 2G SNP (3).

While there is evidence that the 2G polymorphism preferentially binds nuclear proteins, and that enhanced binding has an effect on transcriptional activity of the promoter when DNA is in excess, it is questionable whether the transcription competition effect seen in the cotransfection experiments will also be seen in breast cancer cells. However, two studies on Japanese patients suggest that the presence of a single 2G SNP allele in a person's genome may be a risk factor for developing endometrial carcinoma or ovarian cancer, and that having at least a single copy of the 2G SNP promoter leads to higher MMP-1 production in the tumors of these patients (1, 2). To analyze the possible role of the SNP on MMP-1 production in breast cancer I have begun to analyze breast cancer cell lines and normal breast tissue for their SNP genotype, and MMP-1 production levels. All six of the breast cancer lines I have analyzed are homozygous for the 2G SNP, while the normal breast tissue cell line is homozygous for the 1G SNP (table 1). Five of the breast cancer cell lines were analyzed by western blotting for MMP-1 production. Only MDA 231 demonstrated high levels of MMP-1 (figure 3). Three of the lines were also analyzed by northern blotting for MMP-1 RNA, and once again MDA 231 was the only cancer cell line demonstrating MMP-1 production (figure 4). These findings suggest that the presence of the polymorphism is not sufficient for MMP-1 production.

I next used transient transfections to determine if the breast cancer cell lines had the appropriate nuclear environment to see a difference in the transcriptional levels between a 1G promoter and a 2G promoter. Five of the lines were tested with transient transfections of either the 1G or the 2G promoter driving beetle luciferase expression. The only breast cancer cell line that demonstrated a significant increase in the transcriptional activity of the 2G SNP promoter over the 1G SNP promoter was MDA 231 (14 fold, $p = 0.003$, figure 5), the same cell line that expressed the endogenous MMP-1 gene. The differences in transcriptional activity among these 2G homozygous breast cancer cell lines may be due to a lack of appropriate transcription factors. In the future I plan to explore this possibility by using nuclear extracts from the various breast cancer cell lines to look for differential binding when the extracts are exposed to radio labeled 1G or 2G DNA probes in gel shift experiments. In addition I will attempt to identify any proteins binding specifically to the 2G DNA probe with super shift experiments.

While the breast cancer cell lines provided some material for future experiments, it was clear that a larger sample size was going to be necessary to determine whether the presence of MMP-1 promoters with the 2G SNP affects expression of the endogenous gene. To answer this question I have begun to study human foreskin fibroblasts (HFFs). I chose HFFs because we already had a protocol in place for obtaining the cells, they were readily available so I could easily attain a large sample size, and the behavior of the MMP-1 promoter polymorphism in fibroblasts may provide information on the involvement of stromal cells in metastasis. The study design includes harvesting the fibroblasts from foreskins and genotyping these normal cells for the 1G/2G SNP. Then the basal MMP-1 production of the cells is measured by analyzing cellular RNA with northern blotting. In addition the cells are stimulated with fibroblast growth factor (FGF), epidermal growth factor (EGF), and interleukin 1 (IL-1) and the induced MMP-1 expression is measured. Initial genotyping experiments reveal a Mendelian distribution of MMP-1 promoter genotypes with 7 lines homozygous 1G, 5 lines homozygous 2G, and 14 lines heterozygous (table 2). This agrees with our lab's original analysis of the SNP in normal DNA samples obtained from the center for study of human polymorphisms in France (3). Northern blots for MMP-1 RNA were initially used to analyze the level of transcription in HFF lines. All MMP-1 RNA levels were normalized to GAPDH RNA levels.

Currently there are not enough data to make a thorough statistical analysis of MMP-1 gene expression in these cells; however, the preliminary results indicate that there is no difference in the basal MMP-1 transcriptional activity among cells of the various genotypes (table 3). Induced levels of MMP-1 can be compared from experiment to experiment when expressed as fold increase over unstimulated. Once again preliminary results indicate that there is no difference in the level of MMP-1 transcriptional induction among cells of varying genotypes (figure 6). More data are needed to make a definitive conclusion. Additionally, a real-time RT-PCR protocol is being optimized to quantitate the MMP-1 RNA more precisely and to enable a comparison of expression levels among different HFF lines more easily.

I have not begun experiments with mice. I plan to characterize breast cancer cell lines and the 1G/2G polymorphism further before mice are sacrificed for these experiments.

Key Research Accomplishments:

- Subcloned MMP-1 1G and MMP-1 2G promoter into two different expression vectors
- Cotransfected A2058 melanoma cells with MMP-1 1G and MMP 1 2G reporter vectors
- Observed transcriptional competition at the MMP-1 promoter, but only at high concentrations of DNA
- Genotyped 6 breast cancer cell lines, all 2G homozygotes
- Measured the MMP-1 expression in five breast cancer cell lines by western and three by northern, and noted that only one cell line was producing MMP-1 (MDA 231)
- Performed transient transfections on 5 breast cancer cell lines and noted that the increased transcriptional activity of 2G MMP-1 promoter is only present in 1 of the 5 breast cancer cell lines tested (MDA 231)
- Isolated 40 different HFF lines
- Genotyped 25 HFF lines and noted that the MMP-1 promoter SNP genotype demonstrates a Mendelian distribution in HFF lines
- Examined basal and FGF, EGF, and IL-1 induced MMP-1 production in 12 HFF lines.

Reportable outcomes:

I have no reportable outcomes at this time.

Conclusions:

The data obtained indicate that promoters containing the 2G polymorphism can interfere with transcriptional activity of promoters containing the 1G polymorphism, but only at high concentrations of DNA. The effect of the 2G polymorphism on transcription observed in transient transfections of A2058 melanoma cells is only observed in the breast cancer cell line expressing endogenous MMP-1. The presence of the MMP-1 polymorphism genotype does not affect MMP-1 expression in breast cancer cell lines cultured in vitro.

The data to date do not indicate a role for the MMP-1 promoter polymorphism in expression of the endogenous gene in breast cancer cells; however there have not been enough samples analyzed to definitively make this conclusion. To better address the problem, more samples are needed, and a source of those samples is human foreskin fibroblasts (HFFs). Initial experiments in HFFs have begun, but the amount of data currently limits statistical analysis. Future experiments include real time RT-PCR assays to better quantitate MMP-1 RNA in at least 50 HFF lines.

References:

1. Y. Nishioka *et al.*, *Jpn J Cancer Res* **91**, 612-5. (2000).
2. Y. Kanamori *et al.*, *Cancer Res* **59**, 4225-7. (1999).
3. J. L. Rutter *et al.*, *Cancer Res* **58**, 5321-5. (1998).

Appendices:

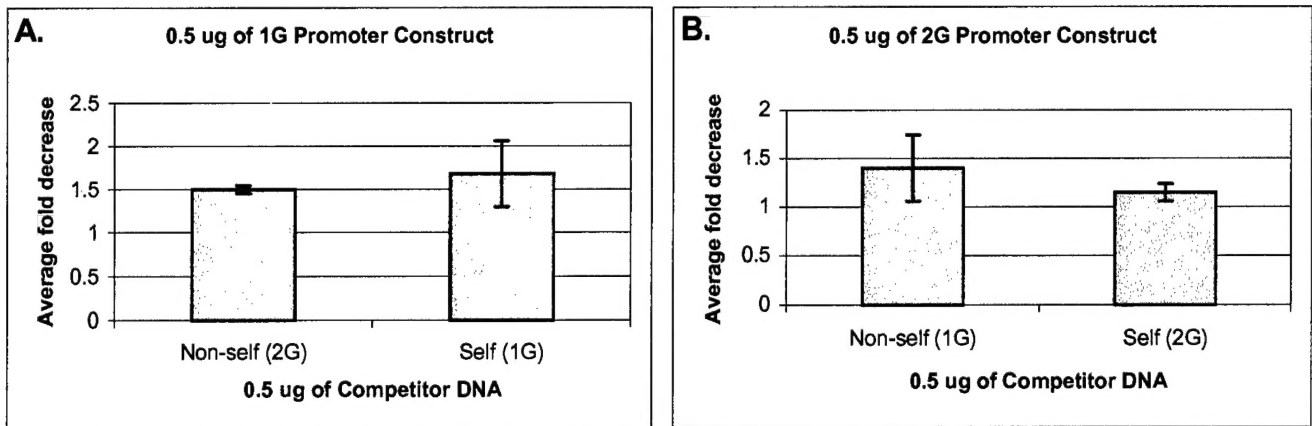


Figure 1. There is no difference in the ability of self or non-self promoters to interfere with transcriptional activity of either 1G or 2G promoters when cotransfected at lower concentrations of DNA. A, a vector containing 1G MMP-1 promoter driving beetle luciferase expression was cotransfected with equal amounts of vectors containing either 2G or 1G promoters directing sea pansy luciferase expression. Measurements of the decrease in transcriptional activity of the 1G promoter in the presence of 1G or 2G competitors is reported. B, measurements of decrease in transcriptional activity from 2G-luciferase constructs when either 1G or 2G competitor are present.

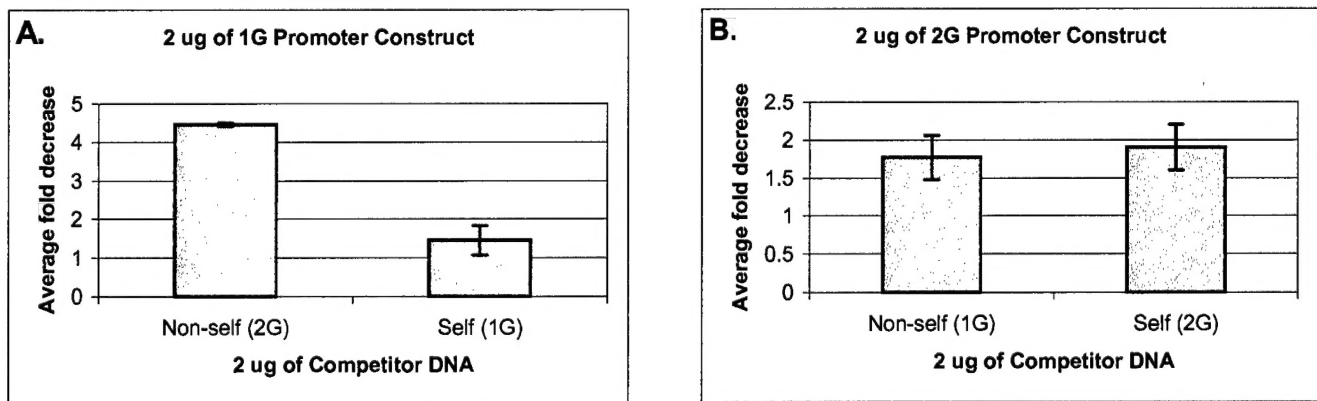


Figure 2. MMP-1 promoters containing the 2G polymorphism can interfere with transcription from promoters containing the 1G polymorphism when cotransfected at higher concentrations of DNA. A, There was a significantly ($p=0.026$) greater decrease in transcriptional activity from 2 μ g of the 1G promoter when an equal amount of 2G competitor was present compared to self-competitor. B, There was no difference in the decrease of transcriptional activity from 2 μ g of 2G promoter when equal amounts of self or non-self competitor were added.

Table 1. Single nucleotide polymorphism (SNP) genotypes of breast cancer cell lines.

*MCF 10A is a normal breast cell line.

Cell Line	SNP Genotype present
SKBr3	2G
T47D	2G
MCF7	2G
MDA 231	2G
ZR75	2G
MDA 468	2G
MCF 10A *	1G

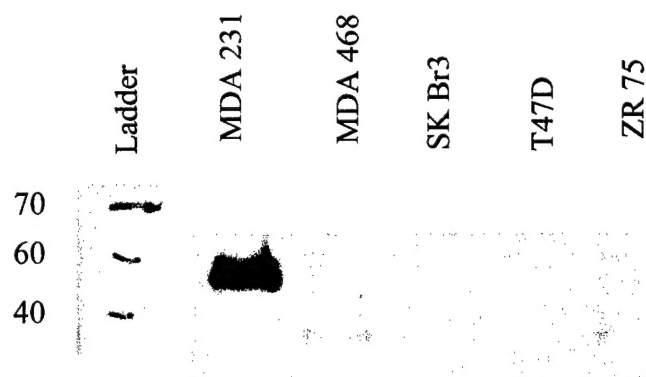


Figure 3. MDA 231 has high levels of MMP-1 protein in serum free culture. Serum free media from 5 breast cancer cell lines were immunoblotted with anti-MMP-1 antibody.

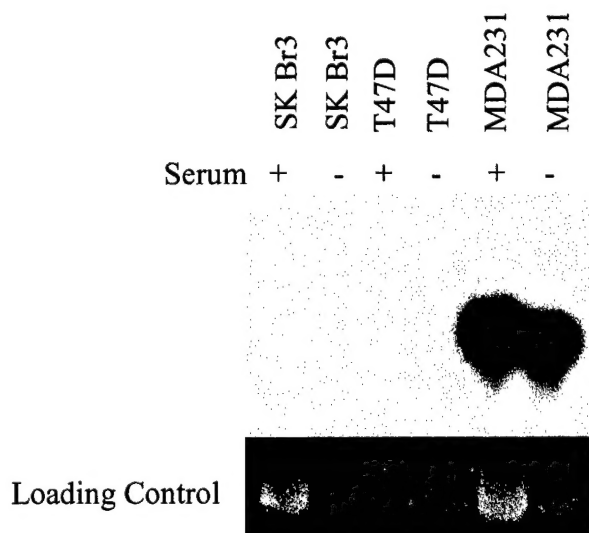


Figure 4. MDA 231 produces high levels of MMP-1 RNA. Northern blots of RNA isolated from three breast cancer cell lines incubated with and without serum revealed that MDA 231 cells produce MMP-1 RNA. The presence of serum does not enhance MMP-1 production.

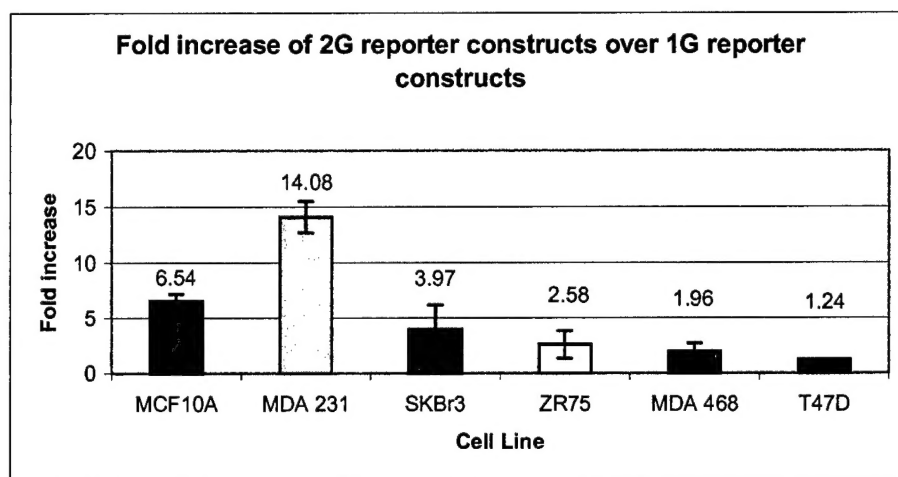


Figure 5. MDA 231 is the only breast cancer cell line that demonstrates a significant increase in transcription when the 2G polymorphism is present. Breast cancer cell lines were transfected with reporter vectors containing the MMP-1 promoter with either the 1G or the 2G SNP. A significant increase in transcription in the presence of the 2G SNP was only seen in the normal breast cell line (MCF 10A, $p=0.013$) or in the MDA 231 breast cancer cell line ($p=0.003$). Paired Student's t-test was performed to determine the significance of the data set for each cell line (SKBr3 $p=0.320$; ZR75 $p=0.292$; MDA 468 and T47D not done).

Table 2. HFF line genotypes.

Genotype	HFF line number
1G homozygote	1, 4, 6, 17, 25, 28
1G/2G	2, 8, 10, 12, 13, 14, 15, 18, 21, 22, 23
2G homozygote	5, 11, 16, 20, 26

Table 3. Basal MMP-1 RNA levels from three different experiments normalized to GAPDH. MMP-1 RNA was measured using northern blotting and a phosphorimager. Numbers reported are relative levels of MMP-1 RNA. Average levels of MMP-1 RNA are reported when possible. Due to differences in the probes used it is only possible to compare basal expression within an experiment.

Experiment	Genotype	RNA Level
1	1G/1G	1.88 \pm 0.63
	1G/2G	1.18 \pm 0.99
2	1G/1G	4.73
	1G/2G	2.14 \pm 2.1
	2G/2G	3.74
3	1G/1G	0.469 \pm 0.56
	1G/2G	0.311 \pm 0.16
	2G/2G	0.31 \pm 0.05

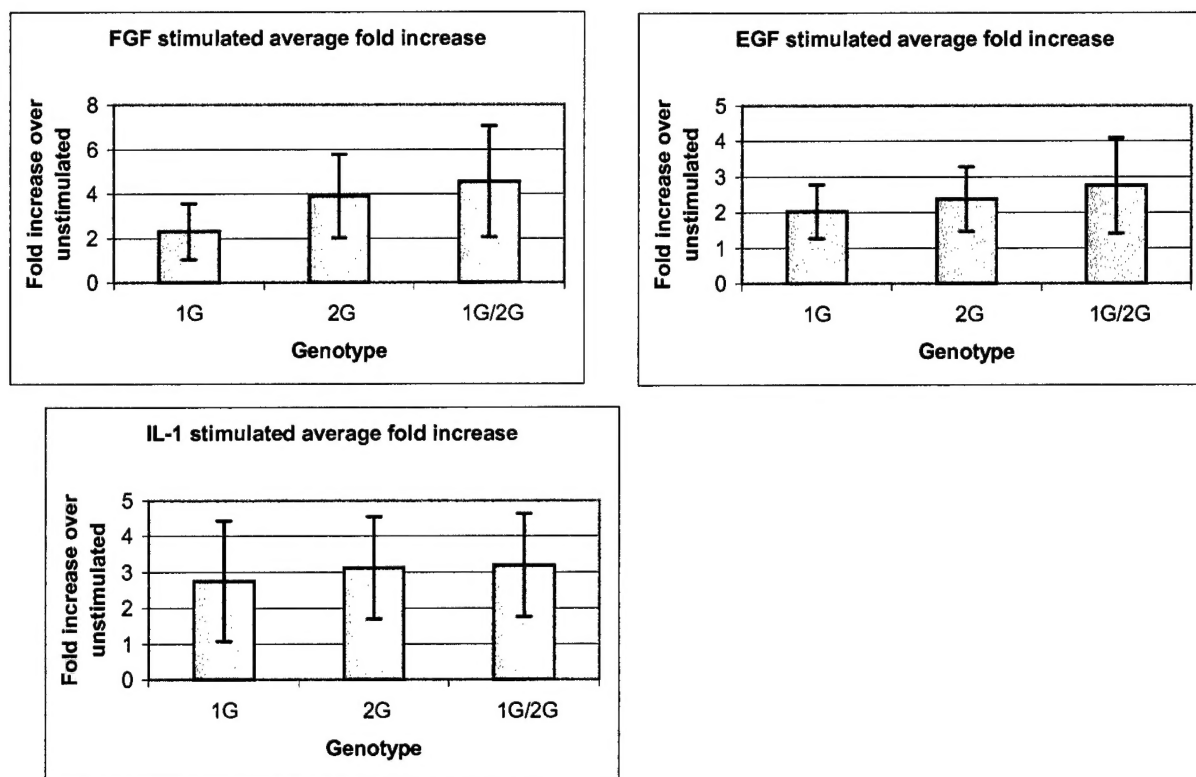


Figure 6. There is no difference in stimulated MMP-1 RNA among SNP genotypes. Preliminary data is reported, and it is insufficient to perform statistical analyses. Cells were stimulated with 10 ng/ml FGF, 10 ng/ml EGF, or 5 ng/ml IL-1.